FORAGE & GRAZING LANDS

Comparison of Laboratory and Quick-Test Methods for Forage Nitrate

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ABSTRACT

Nitrate contained in forage may pose performance and health risks to ruminants. With timely and accurate assessment of forage nitrate levels, steps to reduce the risks of excessive nitrate intake by livestock can be applied. Traditionally, plant nitrate is measured in the laboratory from finely ground oven-dried tissue, which is slower than nitrate quick-test assays of plant sap. However, use of plant sap nitrate requires calibration to nitrate in dried samples. Winter wheat forage (*Triticum aestivum* L.) collected at jointing and heading from fields fertilized with 56 to 235 kg N ha^{-1} was used to compare a laboratory flow injection analysis (FIA) method (Cu-Cd reduction column) with nontraditional laboratory microplate (M-NaR) and field-test (F-NaR) enzyme linked kits (nitrate reductase, E.C. 1.6.6.1), and two hand-held quick-test nitrate assays using a card mounted ion specific electrode (ISE-card) and test strip reflectance meter (TSR). Hot-water extracts of oven-dried samples and fresh samples macerated in propanol solution with a high-speed hand-held blender were prepared. Compared with FIA, mean differences in tissue nitrate were nearly always greater (13–66%, $P = 0.05$) with the other methods. For dried samples, these differences were due partially to extract interferences that suppressed detection of nitrate with FIA and falsely elevated nitrate detection with the ISE-card. Interferences caused only a slight underestimation of forage nitrate with TSR, and were nearly absent with the M-NaR assay. The ISE-card was the most variable and deviated the most from the FIA. Nitrate extraction over a nearly four-fold range was 18% less from fresh than oven-dried tissue. The quick-test consumable cost per nitrate assay was similar for F-NaR and TSR methods, but the TSR was easier to use. Because a hand-held meter is not required with F-NaR, initial startup cost can be reduced. Both TSR and F-NaR performed well for quick-tests of tissue nitrate.

Nitrate risk to ruminants can be affected by various animal factors such as rumen microbes, age and condition, environmental stresses, diet and water quality, as well as several plant factors including nutrient management, species, growth stage, environmental stresses, and nonstructural carbohydrate level (Alaboudi and Jones, 1985; Crawford et al., 1961; Wright and Davison, 1964). Forages containing less than 1000 mg NO_3^-–N kg^{-1} (dry weight basis) usually pose no risk for cattle (Strickland et al., 1995; Undersander et al., 1999). Low levels of ingested nitrate are reduced by rumen bacteria to nitrite and then ammonia (Cowley and Collings, 1977), and any excess ammonia absorbed by the blood stream is excreted in the urine as urea. However, when high levels of nitrate are ingested, the capacity of the normal nitrate conversion process becomes overloaded and a portion of the nitrate is absorbed by the blood stream as nitrate and nitrite. Some of the nitrate that is absorbed recycles back to the rumen through saliva thereby adding again to the nitrate pool in the rumen. In contrast, absorbed nitrite inhibits the oxygen transporting capacity of red blood cells by oxidizing the ferrous iron of hemoglobin to ferric iron (methemoglobin) leading to chronic animal performance problems including suppressed appetite, rate of weight gain, and milk production (Hibbs et al., 1978; Osweiler et al., 1985, p. 460–467; Undersander et al., 1999) and in severe cases, acute toxicity and possibly death. With timely and accurate assessment of nitrate concentration in forage and water sources, potential risks of livestock exposure to excessive nitrate intake may be properly managed or avoided.

Typically, plant tissue nitrate levels increase with increasing amounts of N fertilizer applied to annual cereals and cool-season grasses used as forages (Moeller and Thurman, 1966; Wright and Davison, 1964). In the southern Great Plains, the primary cool-season forage used for stocker cattle (*Bos taurus* L.) enterprises is winter wheat. In many cases, producers grow wheat as a dual-purpose crop for both forage and grain (Pinchak et al., 1996; True et al., 2001). Compared with grain-only and graze-only wheat systems, dual-purpose winter wheat diversifies farming choices and may reduce economic risks (Redmond et al., 1995), but has its own recommended set of management practices to assure success. Dual-purpose and graze-only wheat should be planted earlier than grain-only wheat. To assure early fall growth, fertilizer N needed to achieve a desired grain yield plus additional N to account for N removal in consumed forage is usually applied at planting (Krenzer, 1994; Zhang et al., 1998). Oklahoma grown wheat fertilized with 0 to 168 kg N ha^{-1} had leaf NO_3^-–N values at Feekes growth stage 5 (pseudostem strongly erect [Large, 1954]) that ranged from 99 to 7960 mg kg^{-1} (Raun and Westerman, 1991). At this growth stage, two of the four Oklahoma site-year combinations fertilized with 90 kg N ha^{-1} had leaf NO_3^-–N values exceeding a potentially risk onset level of 1000 mg kg^{-1}. These nitrate levels were present at a time shortly before cattle would normally be pulled-off for dual-purpose wheat production.

Nitrate in plant tissue is readily water-soluble and is most often extracted from samples that have been oven-dried and finely ground (Anderson and Case, 1999). In

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Abbreviations: FIA, flow injection analysis; F-NaR abs, field nitrate reductase kit used with spectrophotometer absorbance detection; F-NaR vis, field nitrate reductase kit used with visual reading; ISE-card, ion specific electrode card; M-NaR, laboratory microplate nitrate reductase kit; NaR, nitrate reductase; TSR, test strip reflectometry.

some cases, fresh tissues are pressed to release sap for nitrate analyses; less often, fresh field samples are extracted directly for nitrate. Relating nitrate in tissue of fresh plant samples to that of dried samples requires development of equations that relate expressed sap nitrate to dried tissue nitrate (e.g., Delgado and Follett; 1998; Errebhi et al., 1998; Hartz et al., 1993; Westcott et al., 1993, 1998; Zhang et al., 1999) or measurement of the water content of the plant sample. Accounting for differences in water content of plant samples should remove variability between sap and dry tissue measurements of plant nitrate (Delgado and Follett, 1998). Once the tissue extract is obtained, the analysis for nitrate should not be delayed unless the extract is chemically preserved to prevent changes in nitrate concentration as a consequence of microbial activity.

There are many methods available for quantifying nitrate in biological and environmental samples. Among the more popular and highly sensitive methods are those that chemically convert nitrate to nitrite followed by a Greiss-Ilosvay chemical reaction to form a pink-purple colored azo dye compound (Sah, 1994). Manual and automated methods using a diverse range of microbial and plant sources of enzyme that biochemically reduce nitrate to nitrite have been developed (Granger et al., 1996; Küche and Schnug, 1996; Lowe and Gillespie, 1975; Lowe and Hamilton, 1967; Patton et al., 2002; Titheradge, 1998), and are most commonly applied for clinical diagnostics. The intensity of an azo dye compound formed with nitrite is proportional to the amount of nitrate and is measured using instruments with absorption or reflection spectrophotometers. Another common method is potentiometer detection of nitrate using an instrument equipped with an ion electrode specific for nitrate (Anderson and Case, 1999; Sah, 1994; Wilhelm et al. 2000).

Some nitrate assay methods have been adapted for field quick-test of water, soil, and plant samples using small hand-held instruments. Others are being developed and should allow a farm consultant or producer to rapidly perform their own field test of forage nitrate. Before acceptance though, research will be required to demonstrate the accuracy, precision, and user friendliness of the quick-test methods. Among the nitrate quick-test methods, results produced with hand-held instruments using reflectance for water samples (Phillips et al., 1995) and soil extracts (Sims et al., 1995; Wetselaar et al., 1998) and potentiometry for plant sap samples (Rosen et al., 1996) and soil solutions (Hartz et al. 1993; Holden and Scholefield, 1995) compared favorably with results obtained with accepted laboratory methods and instruments. In other cases, amounts of nitrate in plant sap were less with a hand-held reflectance instrument (Schaefer, 1986) and greater with an ISE-card meter (Westcott et al. 1998) when compared with nitrate values obtained with accepted laboratory instruments.

This research compared a standard laboratory nitrate assay protocol for winter wheat forage samples to a nontraditional biochemical-linked laboratory and field-test method, and two quick-test methods that use small hand-held instruments. A field nitrate extraction method suitable for fresh wheat pasture samples was compared with standard laboratory extraction of oven-dried and ground wheat samples to evaluate nitrate extraction efficiency. Finally, the presence of substances interfering with nitrate assays of extracts from oven-dried wheat samples was evaluated by a nitrate standard addition technique.

MATERIALS AND METHODS

Forage samples with a wide range of nitrate concentrations were obtained from field-grown winter wheat fertilized with 56 to 235 kg N ha^{-1}. Except for the range of N fertilizer treatments, recommended cultural practices were followed (Krenzer, 1994). The soil series was a Norge silt loam (fine-silty, mixed, active, thermic, Udic Paleustoll) located at the Grazinglands Research Laboratory near El Reno, OK. On 2 Oct. 2000, the entire field received a broadcast application of 56 kg N ha^{-1} in the form of urea. Cultivar 2174 was planted 19 Oct. 2000. Ammonium nitrate fertilizer (0–180 kg N ha^{-1} in 45 kg increments) was hand broadcast in 6-m^2 plots on 13 Mar. 2001 when wheat plants were at Feekes growth stage 6.

Forage Collection and Processing

Forage samples were collected 2 Apr. 2001 (Feekes growth stage 7) and on 9 and 10 May 2001 (Feekes growth stage 10.1). The first sample corresponds to wheat pasture at early graze-out and the second to wheat with heads exposed and ready to be harvested for hay. On both dates, plants in 0.5 m of row from four separate locations in each plot were cut to a height of 5 cm, put into plastic bags, and placed on ice before returning to the laboratory for separate processing of each sample. Plants from each sample were cut into approximately 1-cm segments, mixed thoroughly, and ≈60 g was put into a paper bag, weighed, dried at 60°C to constant weight for moisture content, then ground in a cyclone mill with a 1-mm screen for dry tissue tests. Twenty g subsamples of the cut tissue segments were put into plastic bags then placed in an ultra-low freezer (−80°C) for later use in fresh tissue tests.

Tissue Nitrate Extractions

Dried samples (≈0.1 g) were weighed into screw-cap test tubes (12 by 125 mm) and extracted in 10 mL of deionized H_2O for 1 h at 98°C with vortex mixing at 10-min intervals. The capped tubes were centrifuged at 3750 × *g* for 10 min then decanted into vials for analysis of the extract.

Ten of the frozen samples, with nitrate contents spanning the range as measured in oven-dried tissue, were selected for extraction. Frozen samples (20 g fresh weight) were thawed and then extracted with 40 mL of a propanol solution (5 mL propanol per 100 mL deionized H_2O) with a Braun Multiquick[1] hand-held blender (MR 550 MCA; The Gillette Co., Boston, MA). Tissue was macerated thoroughly by blending at maximum speed (11 600 rpm) for about 2 min and then filtered through one layer of Miracloth (Biosciences, Inc., La Jolla, CA). The extract was centrifuged at 3750 × *g* for 10 min before decanting and centrifuging 2 mL of the extract at 10 800 × *g* for 10 min. Washed filter paper or an economical microcentrifuge (fixed speed with six place fixed angle rotor for 2.0-mL tubes) could be used to clarify extracts in the field. Results presented are referred to as fresh tissue nitrate, even

[1]Trade names and company names are included for the benefit of the reader and do not imply any endorsement or preferential treatment of the product by the authors or the USDA.

though frozen tissue was used for extraction. Compared with fresh tissue, freezing the sample before extraction results in a 7.5 ± 3.4% difference (mean ± SE, $n = 8$) that was not significant (data not shown).

Nitrate Assays

Nitrate in dried and fresh tissue extract was measured by laboratory and quick-test methods that used chemical or enzymatic (NaR, nitrate reductase, E.C. 1.6.6.1) reduction of nitrate to nitrite, or a nitrate specific electrode. Table 1 lists the methods used and the nitrate standard range used for each method. When it was not possible to perform all assays on the day of extraction, aliquots of the extracts were stored at –80°C until an assay could be completed. Unless otherwise indicated, nitrate standards were prepared from a commercial primary stock solution containing 1000 mg NO_3^-–N L^{-1} (LabChem, Inc., Pittsburgh, PA).

Laboratory Methods

FIA (flow injection analysis). A flow injection analyzer instrument (FlAstar 5010 Analyzer; Foss North America, Inc., Eden Prairie, MN) equipped with a Cu-Cd reduction column was used (AN 62/83; Tecator, 1983). To increase the sensitivity of the instrument, a 200-μL injector loop was used. Extracts of dried tissue were diluted 1:10 with deionized H_2O and extracts of fresh tissue were diluted 1:100 with deionized H_2O.

M-NaR (microplate nitrate test kit). The NaR based Microplate Nitrate Test Kit (M-NTK-301; Nitrate Elimination Company, Inc., Lake Linden, MI) was used according to directions included with the kit. Briefly, in a 96-well microplate, three wells each received 10 μL of nitrate standard or tissue extract (diluted as needed). Dilutions of dried tissue did not exceed 1:3 with deionized H_2O; fresh tissue extracts were diluted 1:20 with extraction solution. Next, 90 μL of an assay buffer mixture was added to each well and the plate was mixed for 20 min, followed by the addition of 30 μL of quench agent to each well and mixing for 10 min. Finally a sequence of two color reagents, each 50 μL, was added to the wells and mixed for 10 min before measuring the 540 nm absorbance of the wells with a SpectraMax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Quick-Test Methods

ISE-Card (compact ion meter). A Cardy compact ion meter (C-141, Horiba Instruments Incorporated, Irving, CA) was used by diluting oven-dried and fresh tissue extracts 1:1 with 150 m*M* $A1_2SO_4$. The meter was calibrated frequently (no more than every three samples) with 35 and 350 mg L^{-1} NO_3^-–N in 75 m*M* $A1_2SO_4$. Each extract was measured three times and the average value was used to compare method results; the electrode surface was rinsed with deionized H_2O between each measurement.

TSR (test-strip reflectance). The Merck RQflex Plus handheld instrument, #16950, and Reflectoquant nitrate test strips, #11697-1 (EM Science, Cincinnati, OH) was used according to directions included with the instrument and test strips. Calibration was achieved with a preprogrammed bar code. Each extract was measured twice using two separate nitrate test strips, and the average value was used to compare method results. Extract from dried tissue was used without dilution and that from fresh tissue was diluted 1:4 with fresh tissue extraction solution.

F-NaR abs and F-NaR vis (field nitrate test kit). The NaR enzyme based multiuse Field Nitrate Test Kit (F-NTK-105; Nitrate Elimination Company, Inc., Lake Linden, MI) was used according to directions included with the kit. Briefly, nitrate standard or tissue extract (dried tissue diluted as needed up to 1:3 with deionized H_2O, fresh tissue diluted 1:20 with extraction solution) was added to a 12- by 75-mm test tube and then assay buffer mixture containing NaR added, followed by mixing and incubation for 15 min. Finally, a mixture of quench agent and color development reagent were added, mixed, and evaluated after 10 min. Nitrate concentration of the extract was either quantitatively measured at 540 nm absorbance (F-NaR abs) of samples and standards in 1-cm path length cells using a SpectraMax Plus microplate spectrophotometer or estimated by visual comparison to a range of nitrate standards (F-NaR vis).

Nitrate Standard Addition

Nitrate assay interference associated with tissue extract was evaluated by adding known quantities of nitrate to an extract from an oven-dried sample. The wheat sample used to prepare the extract contained low nitrate (125 mg NO_3^-–N kg^{-1} as determined by the laboratory M-NaR nitrate assay) and was extracted as described above. A low nitrate wheat sample was chosen so that accurate additions of nitrate to the extract could be made while maintaining the concentration of the extract for the different methods as in the previously described assays of oven-dried tissue. A range of nitrate additions to the extract or deionized H_2O (appropriate for the standard range of each method) was prepared and analyzed for nitrate using FIA, M-NaR, ISE-card, and TSR methods. For each nitrate assay method, the concentrations of extract in the assays were the same or only slightly less than those used with the oven-dried samples. Final concentrations of extract in the assays were 100 mL L^{-1} with FIA, 500 mL L^{-1} with ISE-card, and 950 mL L^{-1} instead of normally undiluted extract used with the M-NaR and TSR methods.

Table 1. Laboratory and quick-test methods and measurement ranges used to assay nitrate in tissue extracts.

Method	Nitrate detection principle	NO_3^-–N standard range
		mg N L^{-1}
Laboratory		
FIA (flow injection analysis)	Cu-Cd column conversion of nitrate to nitrite followed by color reaction and absorbance measurement	0.01–2.0
M-NaR (microplate nitrate test kit)	Enzyme conversion of nitrate to nitrite with color reaction and absorbance measurement using microplate	1–10
Quick-test		
ISE-card (compact ion meter)	Potentiometric ion specific electrode	14–1400
TSR (test-strip reflectance)	Test strip chemical conversion of nitrate to nitrite with color reaction and reflectometry measurement	1.1–50.8
F-NaR abs and F-NaR vis (field nitrate test kit)	Enzyme conversion of nitrate to nitrite with color reaction and absorbance (abs) measurement or visual rating (vis)	1–10

Statistical Analysis

Comparisons of tissue nitrate results for the methods were made by the Fit Y by X Platform and the Matched Pairs Platform of JMP statistical software (JMP version 5.0.1, SAS Institute, 2002). In the Fit Y by X platform, linear regressions were determined by an orthogonal fit to adjust for variability in *X* as well as *Y* variables. The analyses were performed assuming a variance ratio of 1 and confidence limits of the slope calculated for $\alpha = 0.05$. Confidence limits of the *Y*-intercept were estimated by confidence limits of the slope and mean values of the *X* and *Y* variables. Results of the paired *t* test were illustrated with graphics for the Matched Pairs Platform that plots the differences of the two responses on the *y*-axis and the mean of each pair of the responses on the *x*-axis and is equivalent to rotating a scatter plot of the two responses by 45° to the right. The plot shows a horizontal line for the mean difference of the matched pairs bounded by the 95% confidence interval above and below. If the confidence interval region includes the matched pairs mean difference of 0 on the *y*-axis, then the nitrate result obtained by each method are not significantly different at the 0.05 level.

Linear regression with a least squares fit and Matched Pairs *t* test (JMP version 5.0.1, SAS Institute, 2002) were used to evaluate the presence of substances in oven-dried tissue extract that interfered with nitrate assays. For each method the nitrate concentration of an untreated extract from an oven-dried wheat sample was estimated from the intercept of the linear regression of nitrate treated extracts (response variable) with amount of nitrate added to the extract (factor variable). The estimated amount of nitrate in untreated extract was then subtracted from the measured nitrate concentration of treated extract to give adjusted nitrate concentrations representing the different amounts of nitrate standard added to the original extract. The adjusted nitrate concentrations of treated extract were then regressed against the nitrate concentrations of nitrate standards prepared with deionized H_2O. For each method, interferences in the extract were considered present if slopes ± 2SE did not included the value of 1, and the *t* test of the mean difference of matched pairs was significantly different ($P = 0.05$) from 0.

RESULTS

Method Comparisons

The agreement of tissue nitrate values obtained by the M-NaR, TSR, and F-NaR abs nitrate assay methods with the FIA nitrate measurements of extracts from oven-dried tissue was markedly better than that of the ISE-card method (Fig. 1; Table 2). Often the nitrate value of a sample measured by the alternative laboratory and quick-test methods was greater than the value obtained from FIA. In all cases, tissue nitrate mean differences between M-NaR, ISE-card, TSR, and F-NaR abs and the FIA method matched pairs were significantly greater ($P = 0.05$) than 0 (Fig. 1). Nitrate values measured with the ISE-card averaged 90% greater than those obtained by FIA, while the enzyme methods (NaR and F-NaR abs) and TSR method averaged about 26 and 15% greater than the FIA results, respectively (Table 2, slopes). Only the regression of the TSR method with the FIA method had estimated *Y*-intercept confidence limits that bracketed the origin and the *Y*-intercept upper confidence limit values of the other methods were no more than 57 μg NO_3^-–N g^{-1} dry weight less than the origin (Table 2).

Fresh tissue nitrate values measured by all but the F-NaR abs alternative method appeared to exceed the FIA measurements of fresh tissue extracts (Fig. 2), and was confirmed by matched pairs *t* tests. Compared with the laboratory FIA nitrate measurements, the mean difference in matched pairs of fresh tissue nitrate values obtained with F-NaR abs was not significantly different ($P = 0.05$) from 0, whereas the mean differences between matched pairs of the FIA and M-NaR, TSR, and ISE-card nitrate assay methods was greater ($P = 0.05$) than 0 (statistical analyses not shown). The correlation of nitrate values with those measured by FIA were equally strong with the TSR quick test and the laboratory M-NaR nitrate assay methods ($r = 0.99$, Table 3) but less so with the ISE-card ($r = 0.94$, Table 3). Only the ISE-card nitrate assay method appears to have a slope not different from 1.0 (slope ± confidence limits, 1.250 ± 0.328) within the 500 to 1500 μg NO_3^-–N g^{-1} dry weight range obtained using extracts of fresh tissue (Table 3). Estimated *Y*-intercept confidence limits of the M-NaR method bracketed the origin, while the *Y*-intercept lower confidence limit of the ISE-card method was slightly greater than 0 and the upper confidence limits of the TSR and FN-NaR were slightly less than 0 (Table 3).

Tissue nitrate values obtained with the quick-test F-NaR method using a spectrophotometer (F-NaR abs) and visual rating (F-NaR vis) of the nitrate assays for extracts from both oven-dried and fresh field samples were correlated strongly ($r \geq 0.95$) and had a correspondence not significantly different from 1:1 (Fig. 3). The tissue nitrate mean difference of matched pairs was not significantly different ($P = 0.05$) from 0 with extracts from oven-dried samples, but the mean difference (187 μg NO_3^-–N g^{-1} dry weight) between the visual and absorbance assays of nitrate using fresh tissue extracts was significantly greater than 0 (Fig. 3, matched pairs *t*-test plot insets). With dry tissue extracts, the estimated *Y*-intercept confidence limits bracketed the origin while the *Y*-intercept lower confidence limit of fresh tissue extracts was only 19 μg NO_3^-–N g^{-1} fresh weight greater than the origin.

Nitrate Extraction Efficiency of Fresh Tissue

Mean differences between matched pairs of nitrate values obtained with fresh tissue extracts and those obtained with oven-dried tissue extracts were significantly less ($P = 0.05$) than 0 for both FIA and M-NaR laboratory nitrate assay methods (data not shown). Nitrate extracted from fresh tissue averaged about 18% less than that recovered from the same tissue that was oven-dried, and the correlations ($r \geq 0.99$) between nitrate values derived from oven-dried and fresh tissue extracts were equally strong when measured by either the FIA or M-NaR laboratory methods within a range of about 500 to 2000 μg NO_3^-–N g^{-1} dry weight of wheat tissue (Fig. 4). Estimated *Y*-intercept confidence limits bracketed the origin for both the FIA and M-NaR laboratory nitrate assay methods.

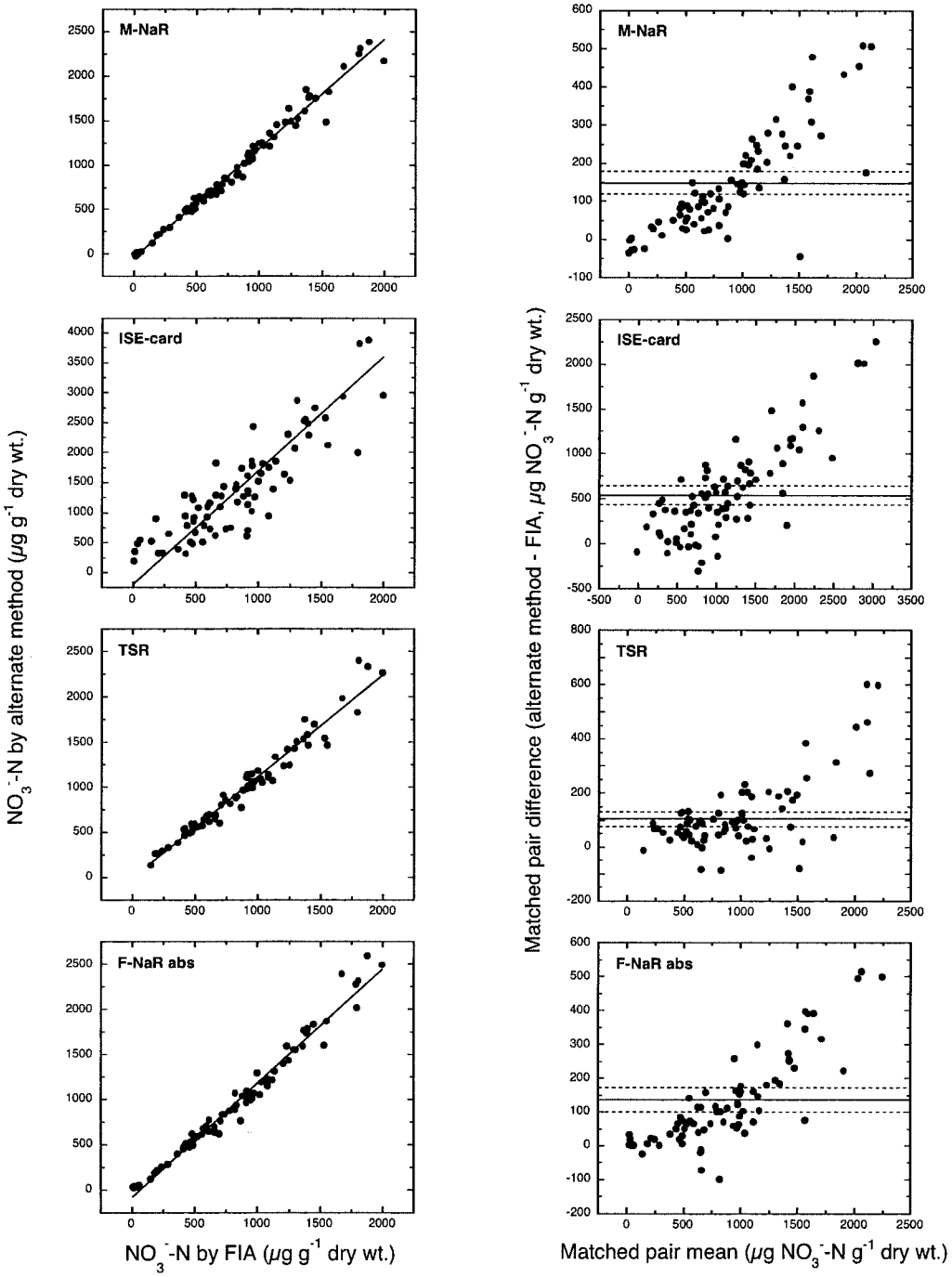

Fig. 1. Scatter plots with linear regression lines (orthogonal fit) and paired *t*-test plots of NO_3^-–N (μg g^{-1}) extracted from oven-dried winter wheat samples (Feekes growth stage 7 and 10.1) and measured by flow injection analysis (FIA), laboratory microplate nitrate reductase kit (M-NaR), ion specific electrode card (ISE-card), test strip reflectometry (TSR), and field nitrate reductase kit with spectrophotometer detection (F-NaR abs). In the panels on the right, mean differences are shown as the horizontal solid lines, with the 95% confidence interval above and below depicted as dashed lines.

Recovery of Nitrate Added to Tissue Extract

For the M-NaR method, concentrations of nitrate added to an extract from an oven-dried sample was nearly identical (1.5% less) to those of nitrate solutions prepared with deionized H_2O, while the concentrations of nitrate in treated extract were almost 25% less than those in deionized H_2O when measured by FIA (based on linear regression slopes, Fig. 5 insets). The TSR assays of nitrate concentration in treated extract were also less (14%) than the nitrate concentrations of solutions made with deionized H_2O. In contrast, measurements of nitrate treated extract by the ISE-card method were slightly more than 5% greater than the concentration of nitrate in deionized H_2O (Fig. 5). For each method the nitrate concentration mean difference between matched pairs of treated extract and nitrate solu-

Table 2. Parameter estimates for linear regressions (orthogonal fit), confidence limits (CL) of the slopes and *Y*-intercepts (α = 0.05), and correlation coefficients of a laboratory and three quick-test assay methods with respect to a flow injection analysis laboratory method used to measure NO_3^-–N concentration of wheat samples using extracts of dried tissue. Parameters are for regression lines depicted in Fig. 1.

Alternative method†	Slope	Slope CL	*Y*-intercept	*Y*-intercept CL	*r*
M-NaR	1.245	±0.038	−52.9	±31.7	0.99
ISE-card	1.900	±0.216	−196.3	±178.7	0.88
TSR	1.154	±0.054	−30.2	±48.0	0.98
F-NaR abs	1.283	±0.048	−96.5	±39.8	0.99

† M-NaR, laboratory microplate nitrate reductase kit; ISE-card, ion specific electrode card, TSR; test strip reflectometry; F-NaR abs, field nitrate reductase kit (spectrophotometer reading).

tion made with deionized H_2O was significantly different ($P \leq 0.05$) than 0 (Fig. 5 insets).

DISCUSSION

Tissue nitrate values determined by FIA to assay nitrate in extracts of both oven-dried and fresh winter wheat samples were consistently less than those of the M-NaR laboratory nitrate assay method and all of the nitrate quick-test methods used in this study. Results from the NaR kits were strongly correlated with those obtained by the TSR method; for oven-dried samples $r \geq 0.98$ and for fresh samples $r \geq 0.99$ (data not shown). Even though the matched pair mean differences in tissue nitrate between the NaR methods and the TSR method were significantly greater ($P = 0.05$) than 0, this difference did not exceed 56 μg NO_3^-–N g^{-1} dry weight (data not shown) and was less than the matched pair mean differences (>137 μg NO_3^-–N g^{-1}) between the NaR methods and the FIA methods (Fig. 1). Similarly, for extracts of fresh tissue (20 g fresh weight per 40 mL of dilute propanol solution), assays of nitrate using FIA (1:100 dilution of extract) were usually lower than the assays of nitrate with the other methods (Fig. 2).

Because the efficiency of nitrate reduction by the Cu-Cd reduction can be adversely affected by interferences in plant extracts (Alves et al., 2000; MacKown, unpublished data, 1990), we have routinely used a dilution ratio of no less than 1:10 when nitrate is measured in extracts of oven-dried plant samples (0.1 g 10 mL^{-1} deionized H_2O). Apparently, the extraction protocols and dilution of extracts used with these wheat samples caused interference in the reduction and assay of extracted nitrate. This was confirmed using an extract of an oven-dried wheat sample from this study to compare the concentrations of nitrate added to the extract with concentrations of nitrate added to deionized H_2O (Fig. 5). The extract imparted only minimal interference with the M-NaR nitrate assay method. The magnitude of deviations between the FIA method and the TSR and NaR kit nitrate assays (Fig. 1) mirrored the differences in response to apparent interferences in the oven-dried sample extract treated with nitrate (Fig. 5). With the ISE-card nitrate assay, measurements of nitrate treated extract from the oven-dried sample were slightly greater than those with nitrate in deionized H_2O (Fig. 5), and would account partially for the differences observed

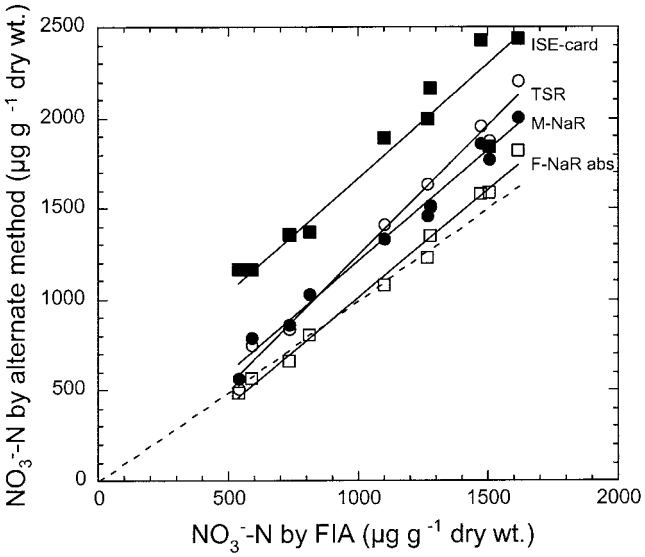

Fig. 2. Laboratory microplate nitrate reductase kit (M-NaR, ●), ion specific electrode card (ISE-card), test strip reflectometry (TSR, ○), and field nitrate reductase kit (spectrophotometer detection; F-NaR abs, □) determinations of nitrate using fresh tissue extracts compared with measurements obtained by flow injection analysis (FIA) using fresh tissue extracts. Winter wheat samples were collected at Feekes growth stage 7 and nitrate concentrations are expressed on a dry weight basis. Diagonal dashed line corresponds to nitrate measurements by alternate methods that would be equivalent with the FIA method ($y = x$).

between the ISE-card nitrate assays and all the other methods (Fig. 1). Apparent underestimation (FIA, TSR) and overestimation (ISE-card) of tissue nitrate values would be important when examining physiological processes of nitrate metabolism of forages. However, in terms of screening for potentially harmful nitrate levels in livestock feeds, forage and livestock management decisions based on the differences in nitrate values among the quick-test methods would probably be unaffected.

Among the alternative methods, the ISE-card was more variable and deviated the greatest from the results obtained using FIA to measure nitrate extracted from wheat samples (Fig. 1, Fig. 2, and Table 3). This occurred even though ionic strength adjustment and frequent two-point calibration of the meter was performed. For assays of nitrate in potato petiole sap, Rosen et al. (1996) obtained excellent agreement of concentrations be-

Table 3. Parameter estimates for linear regressions (orthogonal fit), confidence limits (CL) of the slopes and *Y*-intercepts (α = 0.05), and correlation coefficients of a laboratory and three quick-test assay methods with respect to a flow injection analysis laboratory method used to measure NO_3^-–N concentration of wheat samples using extracts of fresh tissue. Parameters are for regression lines depicted in Fig. 2.

Alternative method†	Slope	Slope CL	*Y*-intercept	*Y*-intercept CL	*r*
M-NaR	1.232	±0.118	−22.6	±128.3	0.99
ISE-card	1.250	±0.328	419.0	±358.0	0.94
TSR	1.436	±0.143	−191.9	±156.3	0.99
F-NaR abs	1.190	±0.097	−179.0	±105.6	0.99

† M-NaR, laboratory microplate nitrate reductase kit; ISE-card, ion specific electrode card, TSR; test strip reflectometry; F-NaR abs, field nitrate reductase kit (spectrophotometer reading).

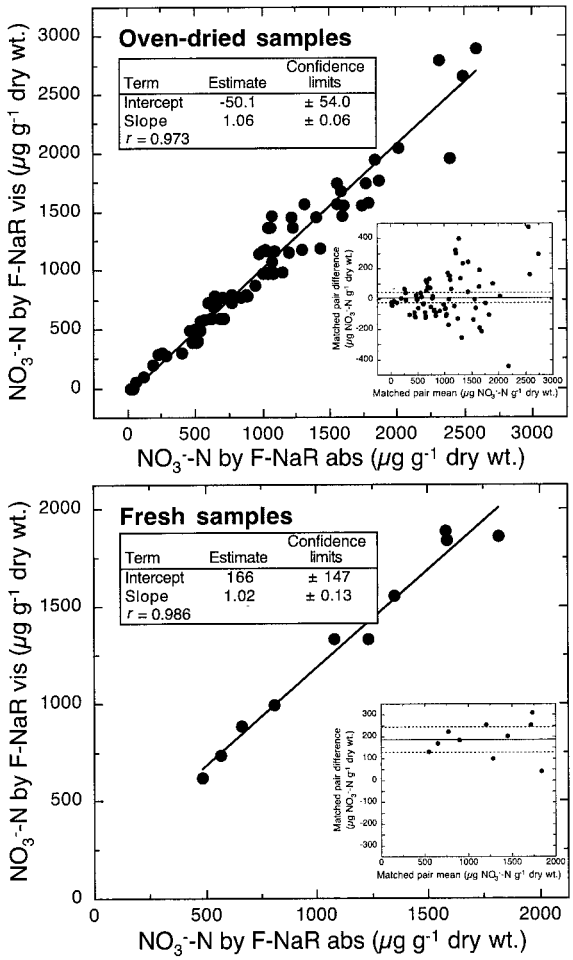

Fig. 3. Comparison of measurements of NO_3^-–N (μg g^{-1}) extracted from oven-dried and fresh winter wheat samples (Feekes growth stage 7 and 10.1) using the field nitrate reductase kit with either visual (F-NaR vis) or spectrophotometer (F-NaR abs) quantification of the Greiss-Ilosvay reaction color. Inset plots depict results of paired *t*-test; the mean difference is shown as the horizontal solid line, with the 95% confidence interval above and below depicted as dashed lines.

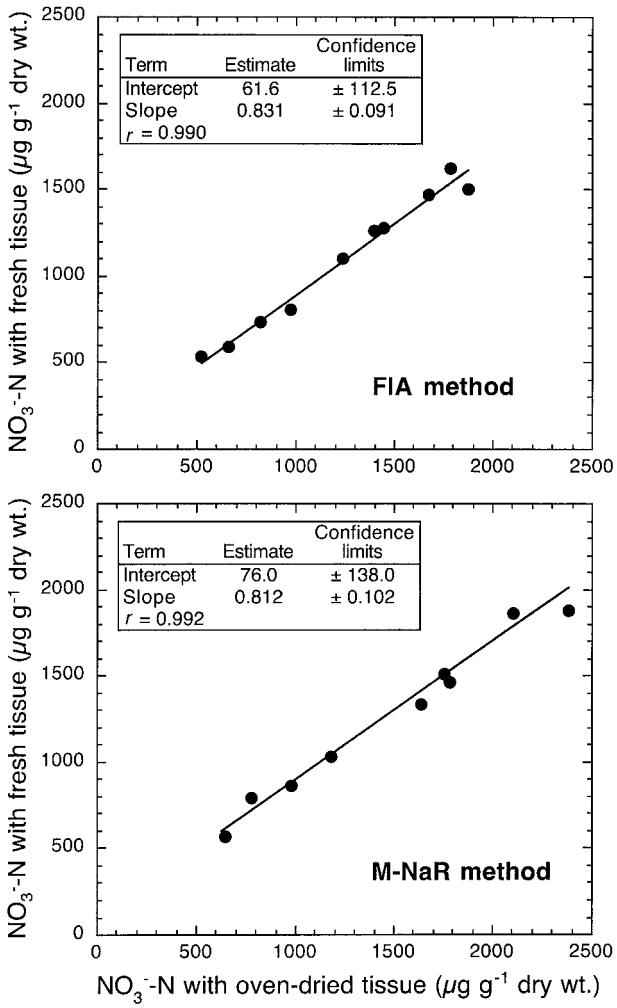

Fig. 4. Relationships between nitrate extracted from fresh and oven-dried winter wheat samples collected at Feekes growth stage 7 and analyzed by flow injection analysis (FIA) and laboratory microplate nitrate reductase (M-NaR) methods. Tissue nitrate concentrations are expressed on a dry weight basis.

tween the ISE-card meter and two laboratory methods (slopes of 1.0 and 0.99), but for sap NO_3^-–N below 750 mg L^{-1} the variability of the ISE-card meter appeared to be greater than that of a laboratory ISE method when both were compared to a laboratory conductimetric instrument that is not sensitive to interferences often observed with ISE measurements. In contrast, we obtained higher values using the ISE-card meter when compared with FIA results (Fig. 1 matched pairs mean difference of 548 μg NO_3^-–N g^{-1} dry weight) and the other methods (data not shown). Westcott et al. (1998) also found that oat (*Avena sativa* L) and barley (*Hordeum vulgare* L.) sap nitrate values obtained by ISE-card meter were 25% greater than those obtained with a laboratory ISE (based on linear regression slope). Among the quick-test nitrate assays the NaR kits and TSR method appeared to be the most accurate with oven-dried tissues (Fig. 1), while with fresh tissue the TSR and M-NaR methods agreed well (Fig. 2).

As conceived, the nitrate extraction protocol for fresh tissue was intended to use a representative subsample (20 g fresh weight) of freshly collected forage and allow rapid extraction with an inexpensive hand-held blender. The moisture content of the forage must be measured or estimated to express fresh tissue nitrate on a dry-weight basis. Relatively rapid gravimetric moisture measurements could be achieved by either conventional or microwave drying. Alternatively, dry matter estimates could be achieved using a developmental growth stage calibration curve that would probably be acceptable for screening forage for potentially toxic levels of nitrate. For example, a fairly precise gravimetric measurement of water content of wheat at Feekes growth stage 7 was obtained in this study with 10 samples of a single variety (mean ± SE, 847 ± 2.7 g kg^{-1} fresh weight). The nitrate extraction protocol used for fresh samples, however, failed to extract nearly 18% of the nitrate found in oven-dried samples (Fig. 4). Apparently the fresh tissue maceration and inclusion of propanol (50 mL L^{-1}) to increase permeability of intact plant cells was less effective for nitrate extraction than achieved with finely-ground oven-dried samples that were extracted with deionized H_2O for 1 h at 98°C. The extraction efficiency

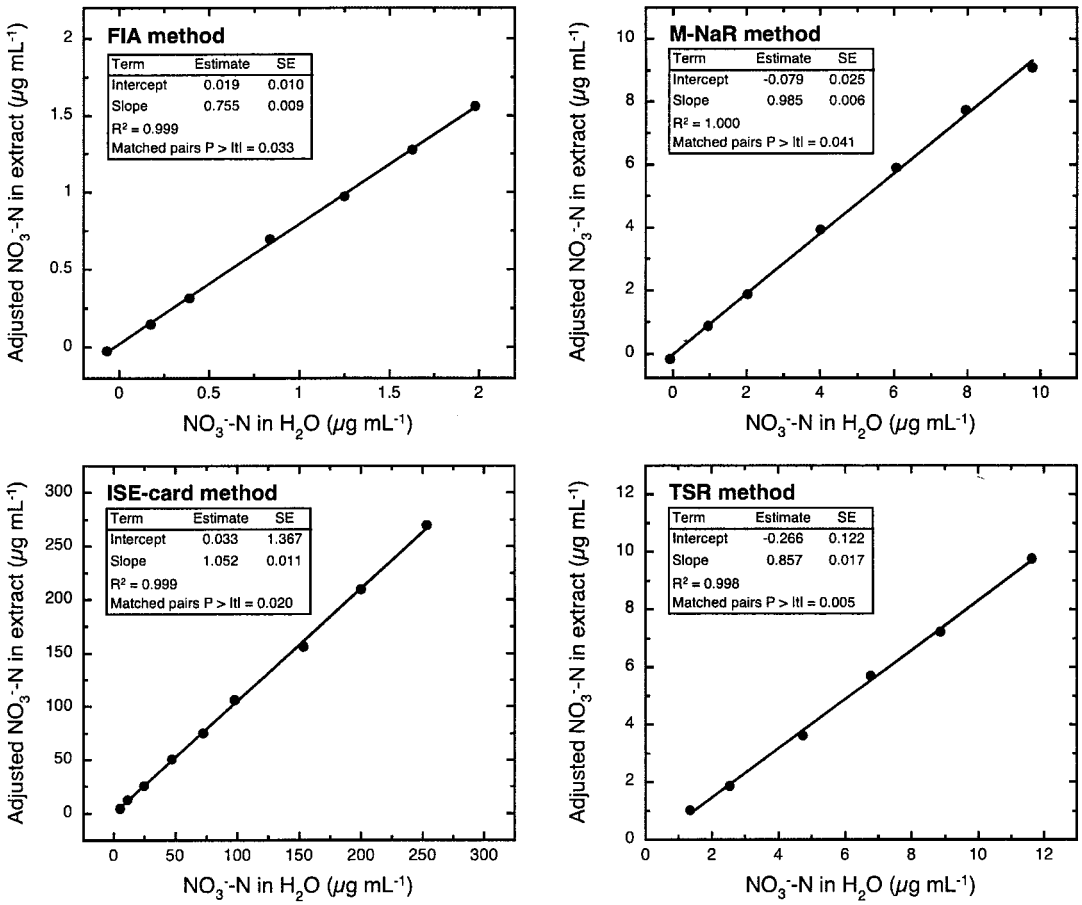

Fig. 5. Linear regression and matched pairs statistical analyses of nitrate additions to an oven-dried tissue extract. For each method (FIA, flow injection analysis; M-NaR, laboratory microplate nitrate reductase kit; TSR; test strip reflectometry; ISE-card, ion specific electrode card), the nitrate concentration of untreated extract was subtracted from the treated extracts and the adjusted nitrate concentrations of the extract regressed against results obtained for nitrate in deionized H_2O.

of fresh tissue nitrate, however, was constant over a nearly four-fold range in tissue nitrate (Fig. 4). To measure nitrate levels of fresh forage accurately, either the efficiency of nitrate extraction must be improved, or the extraction efficiency for each source of forage determined and used to adjust the nitrate level. If a microwave oven were accessible, simply heating the blend of macerated fresh tissue should increase the efficiency of nitrate extraction.

Among the quick-test methods, the consumables cost of 10 nitrate assays ranges from about $3 to $11. The initial cost of an instrument (if required) for the assay can be as much as $1000 (Table 4). The F-NaR field test kit can be used accurately without an instrument, if one visually ranks the nitrate assay with a set of nitrate standards. For nitrate assays of fresh tissue extracts, a nearly 1:1 correspondence (Fig. 3) was obtained between tissue nitrate values derived from visual rankings (F-NaR vis) and those measured with a spectrophotometer (F-NaR abs). The slight overestimation of tissue nitrate (187 μg NO_3^-–N g^{-1} dry weight) obtained with the F-NaR vis nitrate assay method was possibly a consequence of a shift in the hue and saturation color properties of the Greiss-Ilosvay reaction caused by the dark green color in the extract added to the assay reaction mixture.

Selection of a particular nitrate assay method for laboratory or field quick-tests depends on needs for accuracy and ease of use. The laboratory M-NaR method for plant tissue extracts lacks potential interferences and possible health concerns associated with FIA use of a Cu-Cd nitrate reduction column. Although the laboratory M-NaR kit is designed to use microplates for the reaction and absorbance measurements, other alternatives exist. In addition to test tube NaR kits, the enzyme method has recently been developed for water analyses by automated air-segmented continuous-flow instruments, but the cost is currently much greater than with similar instruments using Cu-Cd for nitrate reduction

Table 4. Survey of approximate instrument and consumable costs for quick-test nitrate assays.

Quick-test instrument	Approximate cost of meter	Approximate cost per 10 nitrate assays
ISE-card	$300	$3–$5‡
TSR	$520	$11
F-NaR abs	$340–$1000†	$11§

† Price range from single wavelength filter instrument up to variable wavelength instrument.
‡ Range based on recommended replacement interval of sensor at a cost of $80.
§ For NaR field and test tube assay kits developed and marketed by The Nitrate Elimination Company, Inc., Lake Linden, MI.

(Patton et al., 2002). Additional research will likely document the effectiveness of the automated NaR method for soil and plant extracts. In terms of the quick-test nitrate assay methods for assessment of forage extracts in the field, the cost of the F-NaR abs and TSR methods were similar, but the TSR method was easier to use and appears to be more accurate. Of course, if the initial cost of the TSR instrument is an issue, nitrate assay results obtained with the F-NaR vis method are extremely cost effective and in the hands of an experienced user, essentially equal to those of the F-NaR abs method that requires a portable spectrophotometer. Regardless of the nitrate quick-test method selected, either additional research to improve extraction efficiency of fresh samples is required, or appropriate calibration is necessary to assure accurate field estimation of forage nitrate levels.

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